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CRASP: CFP reconstitution across synaptic partners

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ABSTRACT

Mapping the pattern of connectivity between neurons is widely regarded to be critical for understanding the nervous system. GRASP (GFP reconstitution across synaptic partners) has been used as a promising method for mapping neuronal connectivity, but is currently available in the green color only, limiting its potential applications. Here we demonstrate CRASP (CFP reconstitution across synaptic partners), a cyan-colored version of GRASP. We validated the system in HEK 293T cells, and generated transgenic *Drosophila* lines to show that the system could reliably detect neuronal contacts in the brain. Furthermore, we showed that the CRASP signal could be selectively amplified using standard immunohistochemistry methods. The CRASP system adds to the toolkit available to researchers for mapping neuronal connectivity, and substantially expands the potential application of GRASP-like strategies.

1. Introduction

The brain is composed of enormous number of neurons that are intricately wired with each other, with its functions thought to be largely determined by the connectivity pattern between the individual neurons [1]. Mapping the pattern of connectivity between neurons is therefore critical for the understanding of the nervous system. However, the fine size of neuronal processes and the extreme complexity of the nervous system pose formidable technical difficulties for connectivity mapping. Up to date, *Caenorhabditis elegans* is the only species for which a comprehensive connectivity diagram of its 302 neurons has been obtained using electron microscopy reconstruction methods [2]. However, even with the latest generation of automated electron microscopy image acquisition and reconstruction techniques, these projects remain extremely labor- and time-intensive, requiring millions to even billions of man-hours to complete [3].

GRASP (GFP reconstitution across synaptic partners) is a recently developed strategy [4] that offers a simpler alternative method for mapping neuronal connectivity (Fig. 1A). The strategy is based on the split-GFP methods [5], where the GFP molecule was split into two fragments that were not fluorescent when isolated from each other. Interestingly, when the two complementary parts were in physical proximity, the fragments self-assemble into functional proteins that emitted green fluorescence when excited. In the GRASP strategy, the two fragments of split-GFP were separately expressed on the extracellular surface of two groups of neurons of interest. Green fluorescence could be observed only when these two groups of neurons were in physical contact with each other, providing a measure of whether the neurons are connected. Since its introduction in 2008, GRASP has been widely used for mapping neuronal connectivity in nematodes, fruitflies and mice [4,6–13].

A major limitation of the method, however, is that the system is available in only one color (green) at present. As a result, many useful genetically encoded proteins that emit green fluorescence, such as GFP itself or the calcium indicator G-CaMP [14], could not be used simultaneously with the GRASP system, because signals from the proteins could not be easily separated from each other. We reasoned that such a problem could be overcome by creating variants of GRASP in other colors.

Here we demonstrate a cyan-colored variant of GRASP that we have named CRASP (CFP Reconstitution Across Synaptic Partners). Based on the bright cyan fluorescent protein Cerulean [15], we made five point-mutations to the split-GFP constructs, obtaining a pair of split-Cerulean constructs spCerulean1-10 and spCerulean11.

Abbreviations: GRASP, GFP reconstitution across synaptic partners; CRASP, CFP reconstitution across synaptic partners; HEK, human embryonic kidney; PN, projection neuron; KC, Kenyon cells; IHC, immunohistochemistry.

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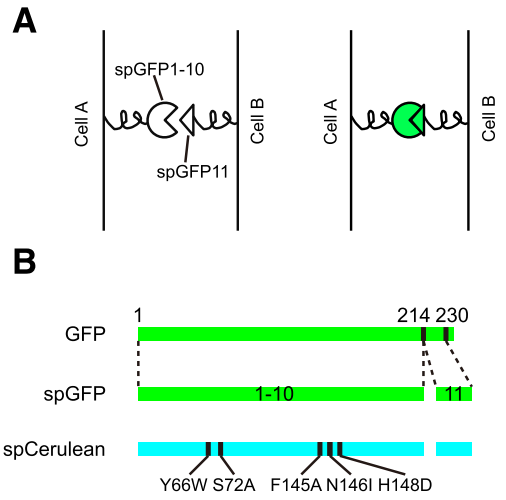

Fig. 1. The GRASP and CRASP system. (A) Schematic illustration of the GRASP system. Green fluorescence could be observed only when cells expressing the two split-GFP fragments are in direct contact with each other. (B) Split-GFP and split-Cerulean. The two split-GFP fragments, termed spGFP1-10 and spGFP11, are evolved from the 1–214 and 215–230 fragments of GFP. Five point mutations were made to spGFP1-10 to obtain spCerulean1-10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We validated that the pair of split-Cerulean reconstitute into functional proteins emitting cyan fluorescence in HEK 293T cells. We then generated transgenic *Drosophila* lines carrying the constructs, and showed that the CRASP strategy could be used to detect neuronal contacts between synaptic partners *in vivo*. Furthermore, we found that the reconstituted CRASP signal could be selectively amplified with immunohistochemistry (IHC). The CRASP system expands the toolkit available to researchers for mapping neuronal connectivity, and opens the possibility for a number of new applications.

2. Material and methods

2.1. Molecular biology

2.1.1. pCAG-mCRASP-spCerulean1-10

We designed spCerulean1-10 by making five point-mutations (Y66W/S72A/F145A/N146I/H148D) to spGFP1-10 [4,16]. The spCerulean1-10 fragment was chemically synthesized (GenScript, Nanjing) to replace the spGFP1-10 fragment in paavCAG-post-mGRASP-2A-dTomato (Addgene # 34912) [11] via the BamHI and XhoI sites, generating pCAG-mCRASP-spCerulean1-10.

2.1.2. pCAG-mCRASP-spCerulean11

This construct was modified from paavCAG-pre-mGRASP-mCerulean (Addgene # 34910) [11], which contains the spGFP11 fragment. Because spGFP11 is equivalent to spCerulean11 (see Results), no modification was necessary to the spGFP11 fragment. However, the mCerulean fragment has to be removed to avoid any potential interference of the CRASP signal. This was done by cutting the mCerulean fragment between the BglII and HindIII sites, followed by blunting and ligating the vector.

2.1.3. pUAST-CD4-spCerulean1-10

The spCerulean1-10 fragment was used to replace the spGFP1-10 fragment in pUAST-CD4-spGFP1-10 [9] between the NheI and SalI sites.

2.2. Fly genetics

Transgenic flies carrying UAS-CD4-spCerulean1-10 were generated by embryo injection of pUAST-CD4-spCerulean1-10 following standard procedures. Transgenic lines with strong expression level and minimal leaky expression were chosen for the subsequent experiments.

Flies were reared on standard medium at 25 °C. Fly stocks used include: GH146-GAL4 (II), 247-LexA (III), UAS-CD4-spCerulean1-10 (III), LexAop-CD4-spGFP11 (II), OK107-GAL4 (IV), GH146-QF (II), QUAS-CD4-spGFP11 (II).

Fly genotype for Fig. 3A were (from left to right):

GH146-GAL4/LexAop-CD4-spGFP11; 247-LexA/UAS-CD4-spCerulean1-10

GH146-QF/QUAS-CD4-spGFP11; UAS-CD4-spCerulean1-10/+; OK107-GAL4/+

GH146-GAL4/+; 247-LexA/UAS-CD4-spCerulean1-10

GH146-GAL4/LexAop-CD4-spGFP11; 247-LexA/+

Fly genotype for Fig. 3B were (from left to right):

GH146-GAL4/LexAop-CD4-spGFP11; 247-LexA/UAS-CD4-spCerulean1-10

GH146-GAL4/+; 247-LexA/UAS-CD4-spCerulean1-10

GH146-GAL4/LexAop-CD4-spGFP11; 247-LexA/+

2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were cultured in DMEM (Life Technologies, 11995-065) supplemented with 10% FBS (Life Technologies, 10099-141) at 37 °C under 5% CO_2. Cells were transfected at ~70% confluence with Lipofectamine 2000 (Life Technologies) in 6-well plates according to the manufacturer's protocol. Cells were fixed for imaging 1 day after transfection.

2.4. Immunohistochemistry

HEK 293T cells or *Drosophila* brains were fixed with 4% formaldehyde in PBS for 3 h on ice and blocked with 10% normal goat serum (Vector Labs, S-1000) in 0.3% PBS-Triton. Primary antibodies were rabbit anti-GFP (Invitrogen, A11122) used at 1:1000, mouse anti-GFP (Invitrogen, A11120) and mouse anti-GFP (Sigma, G6539) used at 1:100, stained overnight at 4 °C. Secondary antibodies were Alexa 568 goat anti-rabbit IgG (Invitrogen, A11036) and Alexa 568 goat anti-mouse IgG (Invitrogen, A11031), both used at 1:500 for staining overnight at 4 °C. The specimen were then cleared with SlowFade Gold (Invitrogen, S36936) and mounted for confocal imaging.

2.5. Confocal imaging

A Nikon FN1 confocal microscope with a 25×, NA = 1.1 or a 40×, NA = 0.8 objective was used for imaging. For imaging the CFP fluorescence, we used 440 nm excitation laser light in combination with a 450/50 emission filter. For imaging the GFP fluorescence, we used 488 nm excitation laser light in combination with a 525/50 emission filter. Images were acquired at the resolution of 512 × 512 or 1024 × 1024, with pixel dwell time adjusted to produce images with optimal signal-to-noise ratio.

3. Results

3.1. Design of the CRASP constructs

We chose to develop a cyan-colored version of GRASP, because CFPs not only share close sequence homology with GFP, but also have a distinct spectral profile that could be easily separated from

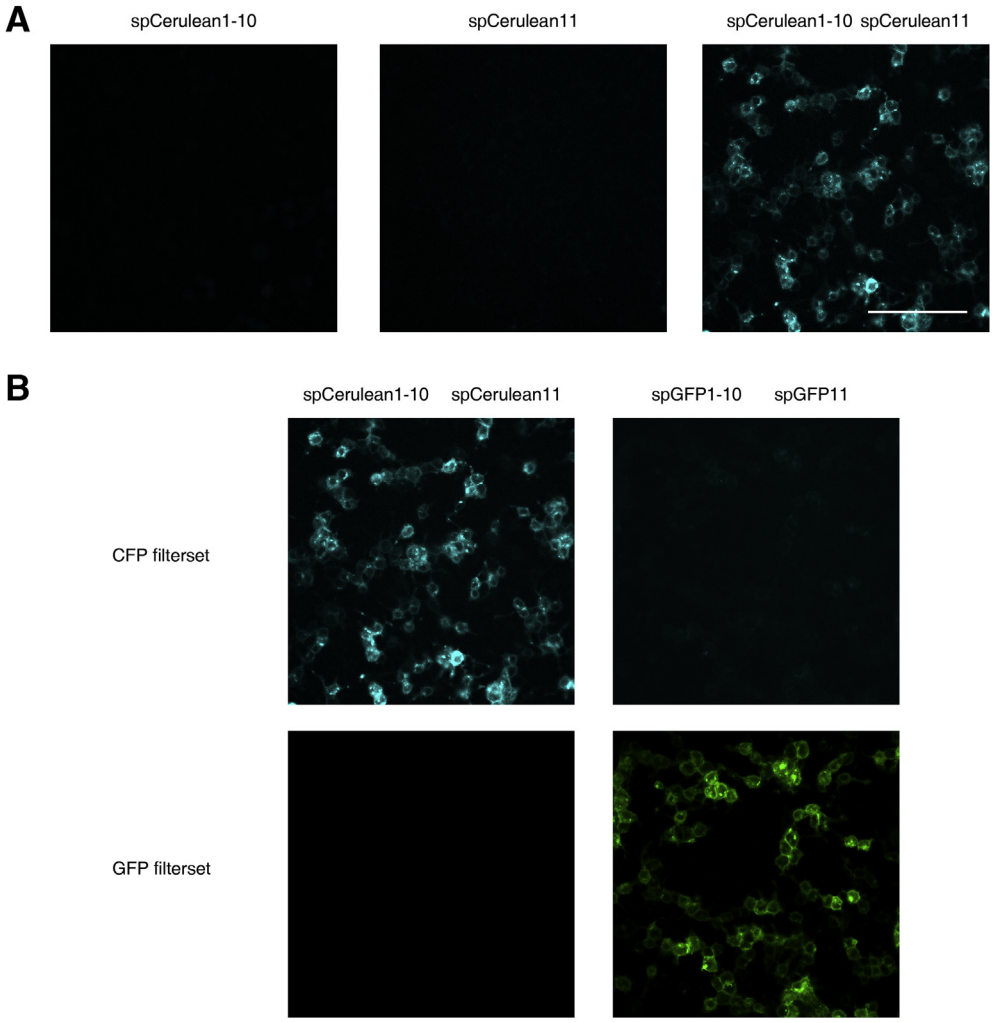

Fig. 2. Validating the CRASP system is HEK 293T cells. (A) Cyan fluorescence was observed when spCerulean1-10 and spCerulean11 were co-expressed but not when either was expressed individually. (B) Reconstituted Cerulean showed distinct spectral profile when compared with reconstituted GFP. Scale bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that of GFP and other fluorescent proteins [17]. We based our design on Cerulean, a bright cyan fluorescent protein that was evolved from ECFP [15]. Incorporating the Cerulean mutations into the corresponding locations of split-GFP [4,16], we constructed spCerulean1-10 with five point mutations (Y66W/S72A/F145A/N146I/H148D) from spGFP1-10 (Fig. 1B). The spGFP11 fragment was used as spCerulean11 with no modification (and referred to interchangeably in this work), because the peptide sequence was identical between GFP and Cerulean in this part.

3.2. Validation of the CRASP system in HEK 293T cells

We first tested whether functional Cerulean proteins could be reconstituted from spCerulean1-10 and spCerulean11 by expressing them in HEK 293T cells. We specifically tethered the spCerulean fragments to the extracellular surface of the cells using a backbone previously used for mammalian GRASP (see Material and methods) [11]. When we transfected cells with only spCerulean1-10 (pCAG-mCRASP-spCerulean1-10), or with only spCerulean11 (pCAG-mCRASP-spCerulean11), no cyan fluorescence could observed. However, cyan fluorescence was observed when the two constructs were co-expressed, indicating that functional Cerulean protein was successfully reconstituted from the two fragments (Fig. 2A). Furthermore, when these cells were imaged using the filter set for GFP, no fluorescence could be observed, demonstrating that the excitation and emission spectrum of the CRASP system was clearly distinct from that of the GRASP system (Fig. 2B).

3.3. Application of the CRASP system to the Drosophila nervous system

We then tested whether the CRASP system could be used to detect neuronal contacts *in vivo*. In *Drosophila*, the connections between the projection neurons (PNs) of the antennal lobe and the Kenyon cells (KCs) of the mushroom body have been widely studied as a model system [18–21]. The axons of PNs made synaptic contacts with the dendrites of KCs only at the calyx area of the mushroom body. Therefore, if the two spCerulean fragments are expressed separately at PNs and KCs, cyan fluorescence should be observed at the calyx area only.

To express the spCerulean proteins on the extracellular side of the membrane, we tethered them to the extracellular domain of human CD4 [4,9]. We generated transgenic flies carrying UAS-CD4-spCerulean1-10, and directed its expression in PNs. Simultaneously, we directed the expression of LexAop-CD4-spCerulean11 in KCs. Consistent to expectation, cyan fluorescence was observed only

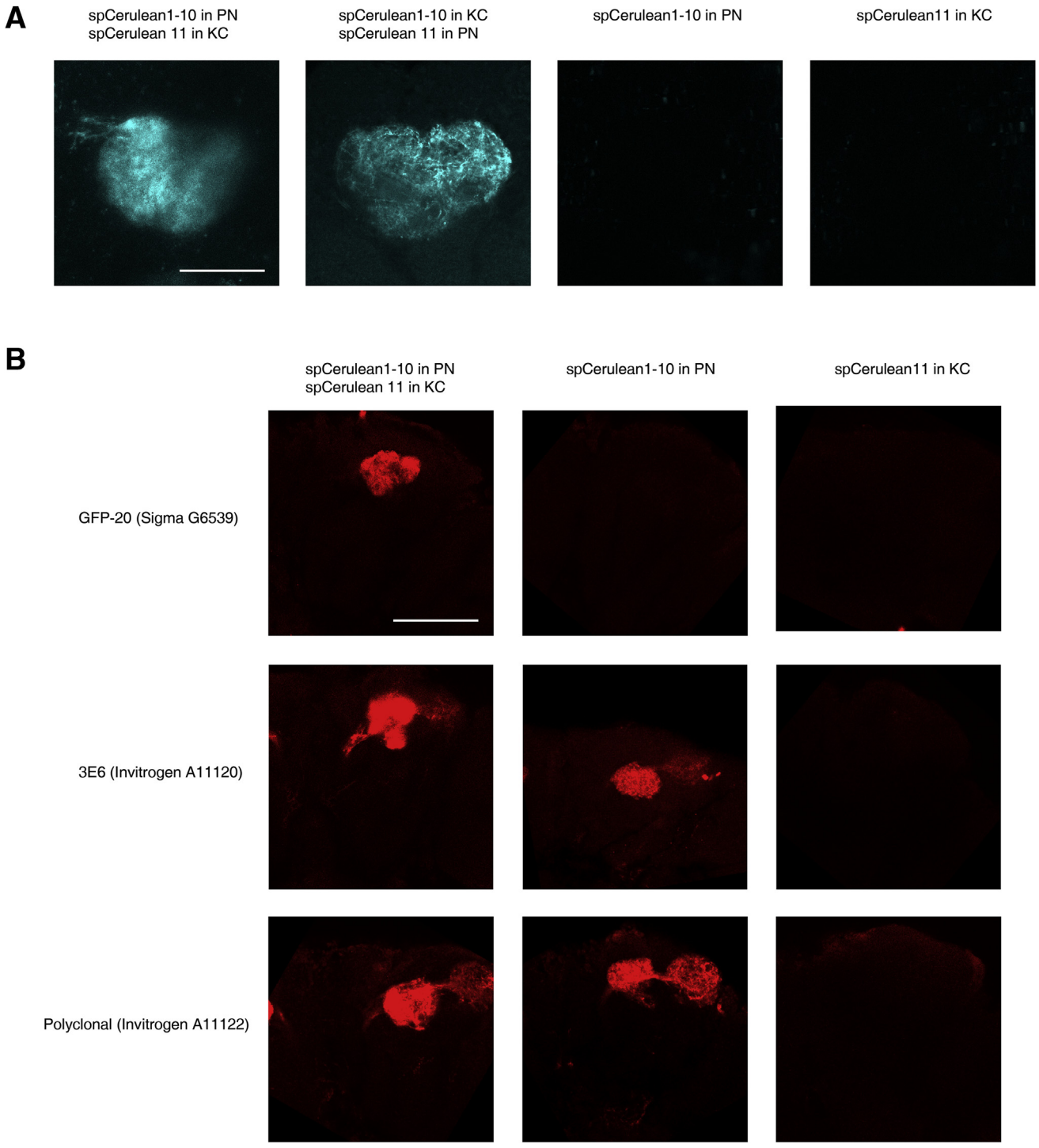

Fig. 3. Application of CRASP to the *Drosophila* nervous system. (A) Cyan fluorescence was observed from the calyx region of the mushroom body when one fragment of split-Cerulean was expressed in the PNs and the other fragment was expressed in the KCs. No fluorescence could be observed when either fragment was missing. Scale bar, 30 μm. (B) CRASP signal could be selectively amplified with the monoclonal antibody GFP-20 but not with two other antibodies. Scale bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from the calyx area of the mushroom body (Fig. 3A). Similar results were obtained when the two fragments were expressed in a reverse manner, with spCerulean1-10 expressed in KCs and spCerulean11 in PNs (Fig. 3A). As a control, when either the spCerulean1-10 or the spCerulean11 fragment was not expressed, no cyan fluorescence was observed (Fig. 3A). Collectively, these results demonstrate that the CRASP system could be used to detect neuronal contacts in intact neuronal circuits in *Drosophila*.

3.4. The CRASP signal could be selectively amplified with IHC

A major problem facing the adoption of GRASP-like strategy is that the reconstituted fluorescence is often weak and somewhat difficult to detect. Although IHC could be potentially used to

amplify the signal, high structural similarity between the spCerulean1-10 fragment and the reconstituted Cerulean necessitates careful screening of the antibodies to be used. It has been previously reported that the monoclonal antibody GFP-20 (Sigma, G6539) selectively recognize the reconstituted GFP and not the split-GFP fragments [9]. We therefore tested this monoclonal antibody, together with two other antibodies, to determine whether the CRASP signal could be selectively amplified. We found that the monoclonal antibody GFP-20 selectively amplified the reconstituted CRASP signal, while another monoclonal antibody 3E6 (A-11120, Invitrogen) and a polyclonal antibody (A-11122, Invitrogen) amplified both the reconstituted CRASP signal and the spCerulean1-10 signal (Fig. 3B). Therefore, the CRASP signal could be selectively amplified using the GFP-20 antibody following routine IHC methods.

4. Discussion

In this study we demonstrated CRASP, a cyan-colored version of GRASP for the study of connectivity between neurons. We showed that reconstituted cyan fluorescence could be observed from the pair of split-Cerulean proteins, both in HEK 293T cells and in *Drosophila* brains. In addition, the CRASP signal could be selectively amplified with routine IHC methods, further increasing the sensitivity of the system.

It should be possible to generate additional color variants of the GRASP system using strategies similar to this work. Yellow fluorescent proteins (YFP) are obvious candidates, although the potential spectral overlap between YFP and GFP is greater than that between CFP and GFP. In addition, a number of red fluorescent proteins and even the photo-switchable protein Dronpa have been reported to be able to reconstitute in a similar manner to split-GFP [22]. However, these constructs were developed for intracellular reconstitution, and further optimization for reconstitution in an extracellular membrane-tethered environment is probably necessary for them [4,16].

With the CRASP system, new potential applications that were previously difficult or impossible for GRASP emerge. For example, CRASP may be combined with GRASP to study the synaptic contacts between more than two groups of neurons. As another example, the widely used genetically encoded calcium indicators G-CaMP [14] or R-GECO [20,23] may now be used simultaneously with the CRASP system, to selectively detect the calcium signals originating from synaptic contacts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.12.011.

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